3D NMR Experiments for Use in Cell Wall Research

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Introduction

NMR (nuclear magnetic resonance spectroscopy) is a powerful tool that has made dramatic impacts into the understanding of cell wall chemistry. Despite the complexity of the wall in general and of the lignin component in particular, intimate structural details are revealed by diagnostic NMR experiments. And now that we understand and routinely apply 1D and 2D NMR (!!??), it seems appropriate to add to the complexity by throwing in a third dimension.

NMR is not limited to 1- and 2-dimensions. Three-dimensional experiments are now commonplace, and 4D-, 5D-, and higher-D-experiments have been applied to labeled proteins. Much of the value of these experiments comes from the further dispersion realized by correlating over the additional dimensions. This is particularly valuable in proteins where ^{13}C, ^{1}H, and ^{15}N dimensions are available. For uniformly ^{13}C-enriched lignins, the 3D HMQC-TOCSY experiment (with one ^{13}C and two ^{1}H axes) has been applied with some success. The increased complexity and data size, and the reduced resolution provide less than compelling advantages over 2D experiments, although their value is becoming appreciated. The expectation that 3D experiments necessarily are more time demanding overlooks the fact that signal-to-noise is gained on the *total number of scans in the entire experiment*. Although labeled materials facilitate 3D (as well as 1D and 2D) experiments, valuable spectra from unlabeled materials are readily obtained.

Results and Discussion

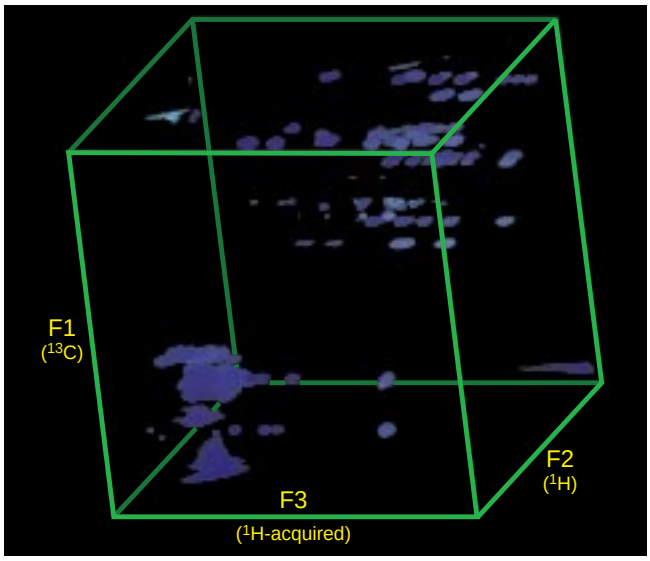
Figure 1a shows a 3D spectrum of the "methoxy-less" lignin, with natural ^{13}C-abundance. In a weekend, this 3D experiment provides ample sensitivity for a synthetic lignin. The spectrum is obtained without applying any of the sensitivity- or resolution-enhancement treatments such as linear prediction in the 3D processing. More informative 2D sub-spectra for the prominent structures in lignins are shown in Figs. 1b–d. Note how "clean" most of these sub-spectra are — a result of dispersion into a third dimension. The particular pulse program used for Figures 1a-e (Bruker's "invbml3d") is not a gradient experiment and has two features requiring comment. Firstly, the sequence, like its 2D-HMQC-TOCSY counterpart ("invbmltp") uses a pre-excitation BIRD sequence to reduce the unwanted intensity from protons attached to NMR-inactive ^{12}C. The BIRD sequence takes about one third of the experimental time; no real data are collected during this time. The other 'feature' is that the directly bonded C–H pair that is excited in the HMQC portion of the experiment retains its $^{1}J_{C-H}$ coupling (~140 Hz). This is illustrated with the slices that are plotted in Figs. 1c-e.

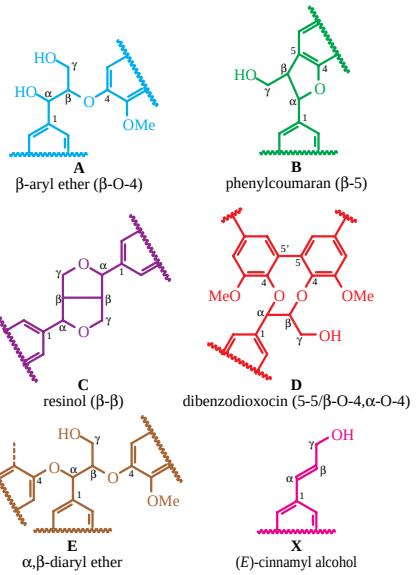
More modern versions using gradient selection decrease the time requirement. A more efficient TOCSY-HSQC experiment has been implemented by Bruker ("mleviietf3gs3d" which can be modified to a two-channel version, "mleviietgs3d") and provides beautiful HSQC sub-spectra in the F_2–F_3 plane, Figs. 1f-k, and TOCSY sub-spectra in F_1–F_3 (not shown). Figures 1h-j show how, if a proton chemical shift is unique, it is possible to observe HSQC spectral planes that are purely from a single substructure in lignin (as seen for structures **A**, **B**, and **C**). Where protons are not unique, HSQC spectra of several units are obtained, such as is the case at 4.45 ppm, where g-protons of substructures **A**, **B**, **C**, **D**, **E** and **X** all resonate, Fig. 1k.

Understanding 3D spectra

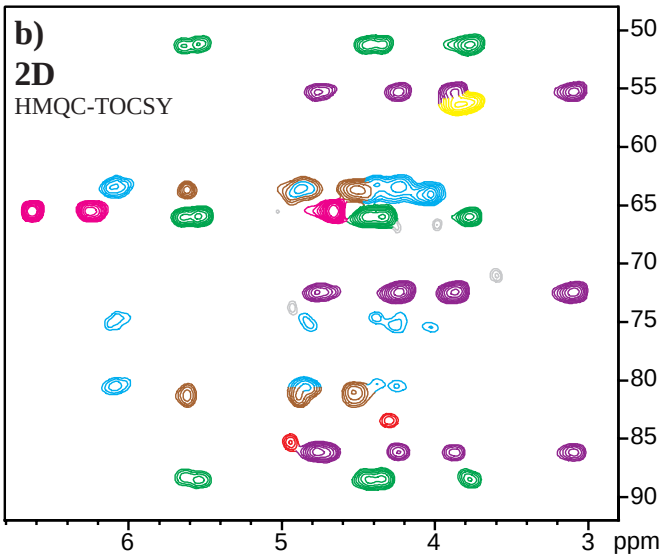
The following brief introduction is provided for those new to the 3D experience. The 3D HMQC-TOCSY experiment is acquired with three orthogonal dimensions, labeled F_1, F_2 and F_3. The acquired dimension is F_3 and, for sensitivity reasons, is proton. F_2 is also proton, and F_1 is carbon. Since this is an HMQC-TOCSY experiment, a 2D F_1F_3 plane is basically a 2D ^{13}C–^{1}H HMQC-TOCSY spectrum at a given proton chemical shift (defined by the distance along the proton F_2 axis). Thus, e.g. Fig. 1d(vii), at the plane through the H–a proton frequency of

a)
DHP
3D HMQC-TOCSY

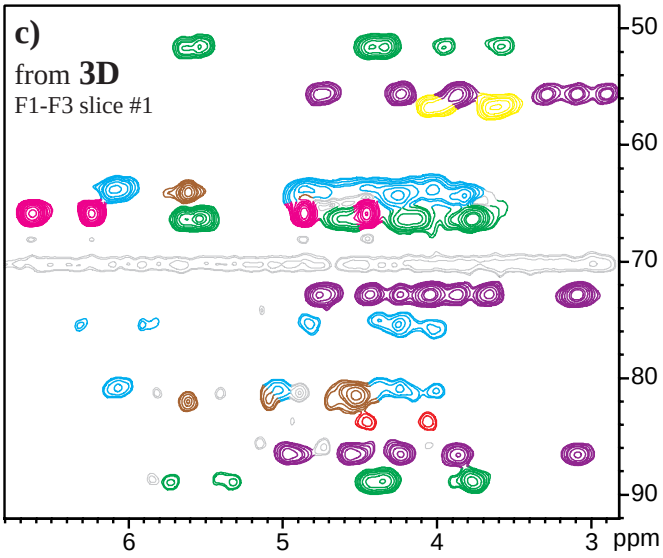




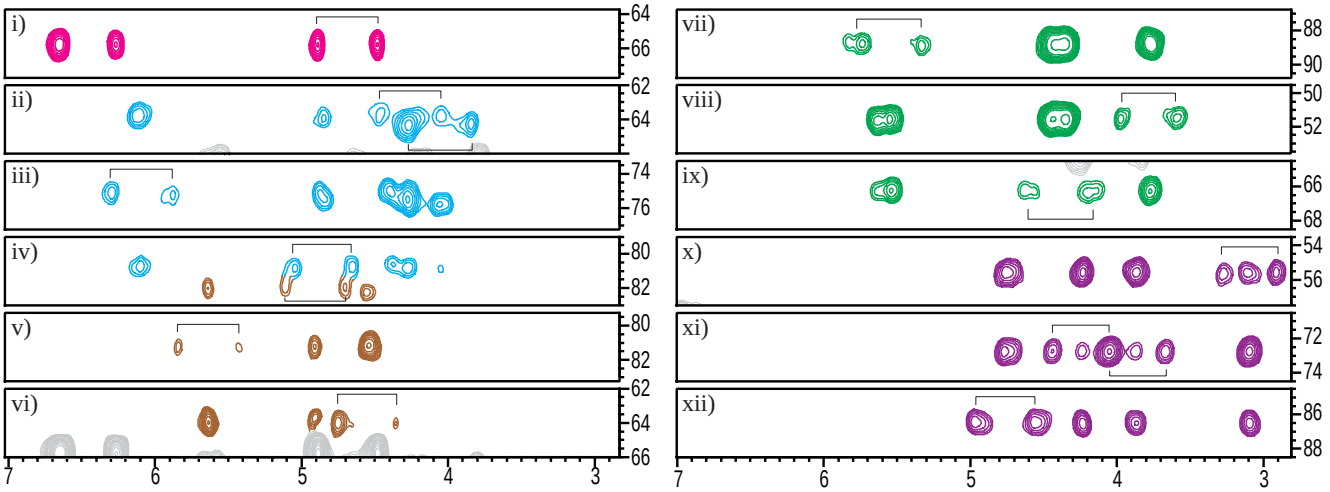
b)
2D
HMQC-TOCSY



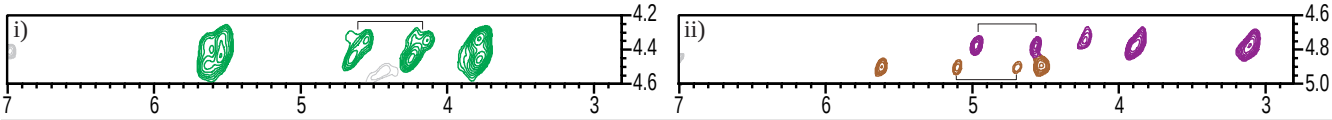
c)
from **3D**
F1-F3 slice #1



d) F1-F3 Slices



e) F2-F3 Slices



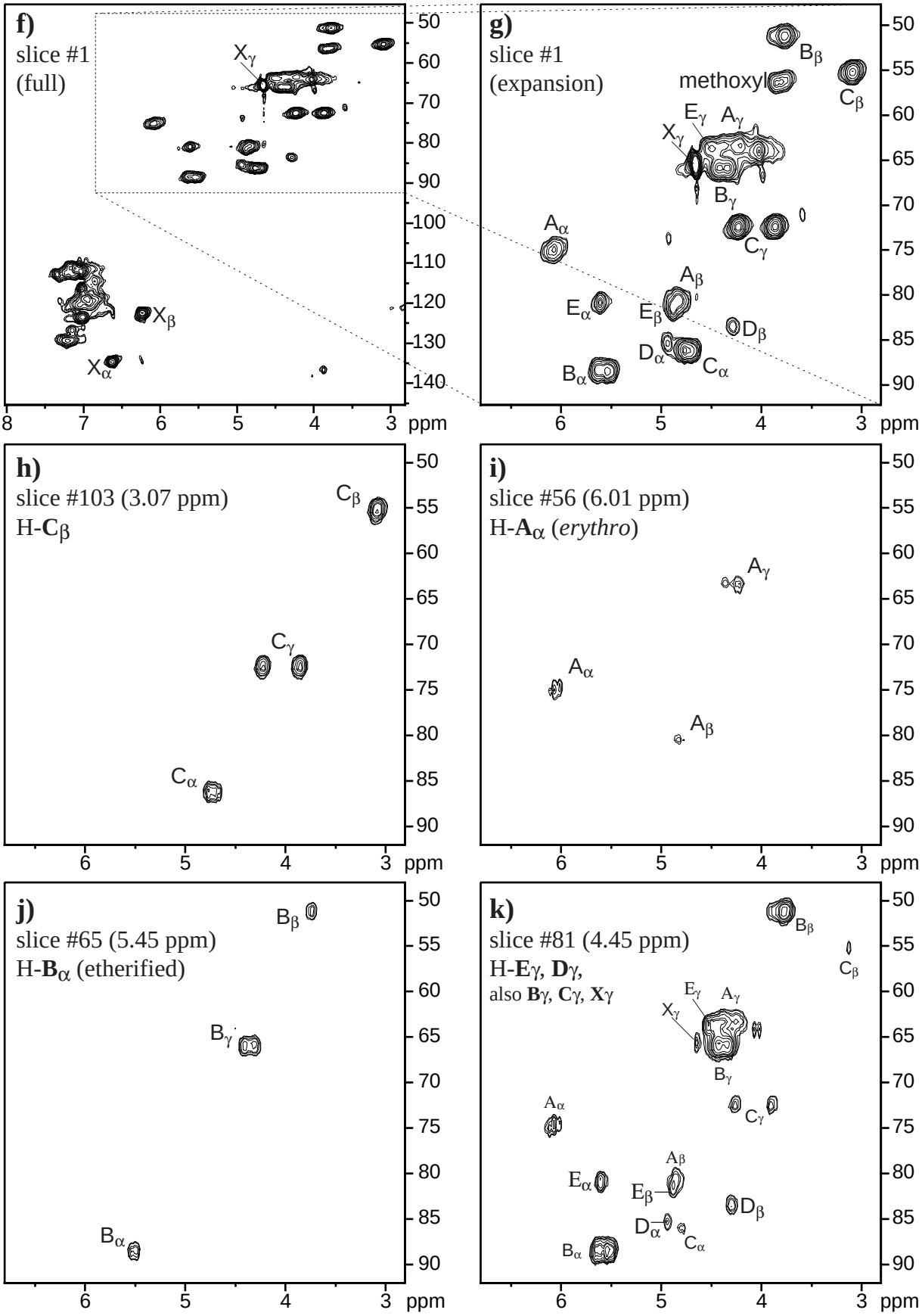

f)
slice #1
(full)
X_γ
X_β
X_α
g)
slice #1
(expansion)
B_β
methoxyl
C_β
E_γ A_γ
X_γ
B_γ
A_α
C_γ
A_β
E_α
E_β
D_β
D_α
C_α
B_α
h)
slice #103 (3.07 ppm)
H-$\mathbf{C}\beta$
C_β
C_γ
C_α
i)
slice #56 (6.01 ppm)
H-$\mathbf{A}\alpha$ (*erythro*)
A_γ
A_α
A_β
j)
slice #65 (5.45 ppm)
H-$\mathbf{B}_\alpha$ (etherified)
B_β
B_γ
B_α
k)
slice #81 (4.45 ppm)
H-**E**γ, **D**γ,
also **B**γ, **C**γ, **X**γ
B_β
C_β
E_γ A_γ
X_γ
B_γ
A_α
C_γ
A_β
E_α
E_β
D_β
D_α
C_α
B_α
ppm

*Figure. 1. a-e: 3D-HMQC-TOCSY experiment (100 ms TOCSY) on a synthetic DHP, at natural abundance. a) presentation of the 3D data-set, rather pretty but spectacularly useless until 2D projections or slices are made for viewing and plotting; b) a 2D HMQC-TOCSY (as seen previously in Fig. 8a) for comparison with the following; c) the first 2D slice in the F_1-F_3 plane is essentially the same as the 2D experiment in b) — differences with this particular experiment are the residual 1-bond ^{13}C–^1H coupling between the excited proton-carbon pair (see text for details); d) F_1-F_3 slices at various proton frequencies (in F_2) showing the beautiful resolution of the major units in HMQC-TOCSY-type sub-spectra; the selected proton corresponding to the slice (e.g. **X**g) is noted; e) two F_2-F_3 slices to show the TOCSY-type data available at selected carbon frequencies (in F_1); the selected carbon corresponding to the slice (e.g. **B**g) is noted. f–g: Gradient-edited 3D-TOCSY-HSQC experiment (100 ms TOCSY) on the same sample, F_3–F_2 slices, 3D plot not shown. f) the first 2D slice in the F_2-F_3 plane is essentially the same as a 2D HSQC experiment; g) expansion of the major sidechain region of slice 1; h) slice through δ_H = 3.07 (H_b of etherified **C** units) showing a pure HSQC spectrum of the sidechain of etherified structures **C**; i) slice through δ_H = 6.01 (H_a of erythro-**A** units) showing a pure HSQC spectrum of the sidechain of erythro-structures **A**; j) slice through δ_H = 5.45 (H_a of etherified **B** units) showing a pure HSQC spectrum of the sidechain of etherified structures **B**; k) slice through δ_H = 4.45 (H_g of structures **A**, **B**, **C**, **D**, **E**, and **X** all overlap) showing composite HSQC spectra of the sidechains of all of these structures.*

phenylcoumaran **B** structures, we see C–a (~88.5 ppm) correlating (in TOCSY fashion) with the H–a (~5.6 ppm, split by $^1J_{Ca–Ha}$), H–b (3.76 ppm), and H–gs (~4.4 ppm). Similarly, a 2D F_2F_3 plane is basically a 2D ^1H–^1H TOCSY spectrum at a given carbon chemical shift (defined by the distance along the carbon F_1 axis). Thus, e.g. Fig. 1e(i), at the plane through the C–gcarbon frequency of phenylcoumaran **B** structures, we see H–a (~5.6 ppm) correlating (in TOCSY fashion) with the H–b (3.76 ppm), and H–gs (~4.4 ppm, split by $^1J_{Cg-Hg}$). Although the profile looks similar, note that the vertical axis of the 2D sub-plot is ^{13}C for Figs. 1d and ^1H for Figs. 1e. The first plane in either dimension is, like in a 2D experiment, very much like a projection of all resonances onto that plane (but with lower signal-to-noise since it represents only a single plane). Therefore, Fig. 1c, the 2D F_1F_3 first plane, is similar to the 2D HSQC-TOCSY spectrum, Fig. 1b (seen previously in Fig. 8). [Unfortunately, we acquired one nasty artifact at about 70 ppm by having the acquired dimension end right on a signal – we were lucky that no other peaks of interest came at a carbon chemical shift of 70 ppm!] See the Web-version at http://www.dfrc.ars.usda.gov for a color version that is easier to interpret!

The 3D gradient-selected TOCSY-HSQC spectrum (3D plot not shown) is understood similarly. In this case, the acquired dimension is again F_3 (proton), but this time F_2 is carbon and F_1 is proton. F_2–F_3 planes are basically 2D ^{13}C–^1H HSQC spectra. The proton shift (on F_1) of the F_2–F_3 plane can be considered as the initially excited proton, which then transfers magnetization to all of the protons in its coupling network. For example, Fig. 1i, an F_1 proton shift of 6.01 corresponds to the a-sidechain proton of b-aryl ether units **A** (in fact just the *erythro*-isomers); that proton is in the same coupling network as the b- and the two g-protons on the same sidechain. During the subsequent HSQC step, each of those sidechain protons correlates with their respective attached carbons. The result in the ideal case is a clean HSQC spectrum of one single unit type, e.g. Figs 1h–j for structures **C**, **A**, and **B** respectively. Such clean spectra derived from individual units in a complex polymer like lignin are an obvious asset for identifying structures —the early lignin structure pioneers could certainly have benefited from such a powerful diagnostic tool. In a similar fashion to F_2–F_3 slices in the non-gradient HMQC-TOCSY experiment described above (Fig. 1e), The F_1–F_3 planes show TOCSY correlations for the proton attached to the carbon at the F_2–frequency of the plane with all of the other protons in the coupling network of that attached proton (not shown).

Conclusions

In the future we will see more and more applications where 3D experiments provide diagnostic information on new structures in lignins. The ability to trace out individual units in the complex polymer is a particularly attractive feature.